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the documents annexed hereto are true copies of:

Application forms P.1 and P.3, provisional specification and drawings of
South African Patent Application No. 2003/6684 as originally filed in the
Republic of South Africa on 27 August 2003 in the name of UNIVERSITY OF
CAPE TOWN for an invention entitled: "A METHOD OF ISOLATING A
THIOL".

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in the Republic of South Africa, this

21 dag van
September 2004
day of

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REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND
ACKNOWLEDGEMENT OF RECEIPT
(Section 30(1) Regulation 22)

REPUBLIC OF SOUTH AFRICA	
FORM P.1	REVENUE
(to be lodged in duplicate)	
27.03.03	R 060.00
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A&A REPUBLIC OF SOUTH AFRICA	

THE GRANT OF A PATENT IS HEREBY REQUESTED BY THE UNDERMENTIONED APPLICANT
ON THE BASIS OF THE PRESENT APPLICATION FILED IN DUPLICATE

21 01 PATENT APPLICATION NO 2003/6684

71 FULL NAME(S) OF APPLICANT(S)

UNIVERSITY OF CAPE TOWN

ADDRESS(ES) OF APPLICANT(S)

Rondebosch 7700, Cape Town, Republic of South Africa

54 TITLE OF INVENTION

A METHOD OF ISOLATING A THIOL

Only the items marked with an "X" in the blocks below are applicable.

☐ THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. The earliest priority claimed is

Country:

No:

Date:

☐ THE APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO 21 01

☐ THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON
APPLICATION NO 21 01

THIS APPLICATION IS ACCOMPANIED BY:

- | | |
|-------------------------------------|---|
| <input checked="" type="checkbox"/> | A single copy of a provisional specification of 16 pages |
| <input checked="" type="checkbox"/> | Drawings of 5 sheets |
| <input type="checkbox"/> | Publication particulars and abstract (Form P.8 in duplicate) (for complete only). |
| <input type="checkbox"/> | A copy of Figure of the drawings (if any) for the abstract (for complete only) |
| <input type="checkbox"/> | An assignment of invention |
| <input type="checkbox"/> | Certified priority document(s). (State quantity) |
| <input type="checkbox"/> | Translation of the priority document(s) |
| <input type="checkbox"/> | An assignment of priority rights |
| <input type="checkbox"/> | A copy of Form P.2 and the specification of RSA Patent Application No |
| <input checked="" type="checkbox"/> | Form P.2 in duplicate |
| <input type="checkbox"/> | A declaration and power of attorney on Form P.3 |
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| <input type="checkbox"/> | Request for classification on Form P.9 |
| <input type="checkbox"/> | Request for delay of acceptance on Form P.4 |
| <input type="checkbox"/> | Extra copy of informal drawings (for complete only) |

21 01

74 ADDRESS FOR SERVICE: Adams & Adams, Pretoria

Dated this 27th day of August 2003

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

The duplicate will be returned to the applicant's address for service as proof of lodging but is not valid unless endorsed with official stamp

REGISTRAR OF PATENTS, DESIGNS, TRADE MARKS AND COPYRIGHT	SPECIAL DATE STAMP
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REGISTRATEUR VAN PATENTE, MODELLE, HANDELSMERKE EN OUTEURSREG	
REGISTRAR OF PATENTS	

(Section 30 - Regulation 8, 22(i)(c) and 33)

PATENT APPLICATION NO		
21	01	2003/6684

A&A Ref: V15902

LODGING DATE	
22	27 August 2003

FULL NAME(S) OF APPLICANT(S)	
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71	UNIVERSITY OF CAPE TOWN
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FULL NAME(S) OF INVENTOR(S)	
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72	STEENKAMP, Daniel Jacobus
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EARLIEST PRIORITY CLAIMED	COUNTRY	NUMBER	DATE
33	Nil	31	Nil

NOTE: The country must be indicated by its International Abbreviation - see schedule 4 of the Regulations

TITLE OF INVENTION	
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54	A METHOD OF ISOLATING A THIOL
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* I/We AMOORE, Hugh

hereby declare that :-

1. ~~I/we am/are the applicant(s) mentioned above;~~
- ** 2. I/we have been authorized by the applicant(s) to make this declaration and have knowledge of the facts herein stated in the capacity of Registrar of the applicant(s);
- *** 3. the inventor(s) of the abovementioned invention is/are the person(s) named above and the applicant(s) has/have acquired the right to apply by virtue of an assignment from the inventor(s);
4. to the best of my/our knowledge and belief, if a patent is granted on the application, there will be no lawful ground for the revocation of the patent;
- **** 5. ~~this is a convention application and the earliest application from which priority is claimed as set out above is the first application in a convention country in respect of the invention claimed in any of the claims; and~~
6. the partners and qualified staff of the firm of ADAMS & ADAMS, patent attorneys, are authorised, jointly and severally, with powers of substitution and revocation, to represent the applicant(s) in this application and to be the address for service of the applicant(s) while the application is pending and after a patent has been granted on the application.

SIGNED THIS 6 DAY OF October 2003

Hugh Amore

Company Name: UNIVERSITY OF CAPE TOWN
Full Names: AMOORE, Hugh
Capacity: Registrar

(no legalization necessary)

- * In the case of application in the name of a company, partnership or firm, give full names of signatory/signatories, delete paragraph 1, and enter capacity of each signatory in paragraph 2.
- ** If the applicant is a natural person, delete paragraph 2.
- *** If the right to apply is not by virtue of an assignment from the inventor(s), delete "an assignment from the inventor(s)" and give details of acquisition of right.
- **** For non-convention applications, delete paragraph 5.

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PRETORIA

FORM P6

REPUBLIC OF SOUTH AFRICA
Patents Act, 1978

PROVISIONAL SPECIFICATION

(Section 30 (1) - Regulation 27)

21	01	OFFICIAL APPLICATION NO
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... 2003 / 6684

22	LODGING DATE
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27 August 2003

71	FULL NAME(S) OF APPLICANT(S)
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UNIVERSITY OF CAPE TOWN

72	FULL NAME(S) OF INVENTOR(S)
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STEENKAMP, Daniel Jacobus

54	TITLE OF INVENTION
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A METHOD OF ISOLATING A THIOL

THIS INVENTION relates to low molecular mass thiols from natural sources. More particularly, the invention relates to a method of isolating thiols.

5 Several protozoal and bacterial pathogens produce low molecular mass thiols which differ from their isofunctional counterparts in mammals, such as, for example, glutathione. These thiol compounds are involved in the detoxification of noxious chemicals, such as are produced in macrophage respiratory burst and in the more prolonged release of nitric
10 oxide by activated macrophages (Nathan C. and Shiloh MU (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc. Natl.Acad.Sci USA*. 97, 8841 – 8). They also play a role (i) in the sequestration of alkylating agents and their conversion to mercapturic acids, which are excreted (Fahey RC (2001) Novel
15 thiols of prokaryotes. *Annu Rev Microbiol*. 55, 33 – 356; Newton GL, Av-Gay Y and Fahey RC (2000) A novel mycothiol dependent detoxification pathway in mycobacteria involving mycothiol S-conjugate amidase. *Biochemistry* 39, 10739 –46), (ii) in providing reducing equivalents for ribonucleotide reductase, a key enzyme in DNA synthesis and consequently cell division

(Dormeyer M, Reckenfelderbaumer N, Ludemann H, and Krauth-Siegel RL (2001). Trypanothione-dependent synthesis of deoxyribonucleotides by *Trypanosoma brucei* ribonucleotide reductase. *J.Biol.Chem.* 276, 10602 – 6; Aslund F, Ehn B, Miranda-Vizueté A, Pueyo C, Holmgren A. (1994) Two additional glutaredoxins exist in *Escherichia coli*: glutaredoxin is a hydrogen donor for ribonucleotide reductase in a thioredoxin/glutaredoxin 1 double mutant *Proc Natl Acad Sci USA* 91, 9813 – 7), and (iii) in the redox regulation of metabolism by modulating the activity of enzymes involved in signalling cascades (Sen CK (2000) Cellular thiols and redox regulated signal transduction. *Curr.Top. Cell Regul.* 36, 1 – 30; Schafer FQ and Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulphide/glutathione redox couple. *Free Radical Biology & Medicine* 30, 1191 – 1212).

Trypanosomatids do produce glutathione, as do most eukaryotes and gram negative bacteria capable of aerobic growth, but then conjugate it to spermidine to form N¹,N⁸-(bis-glutathionyl)-spermidine (or trypanothione), a metabolite thought to only occur in the Kinetoplastida (Fairlamb AH, Blackburn P, Ulrich P, Chait BT and Cerami A. (1985) Trypanothione: a novel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. *Science* 227, 1485 – 1487; Fairlamb AH and Cerami A (1992) Metabolism and functions of trypanothione in the Kinetoplastida. *Ann.Rev Microbiol.* 46, 695 – 729).

Novel thiols have been identified in the Actinomycetes order of bacteria, including in the medically important *Mycobacterium tuberculosis*, the causative agent of tuberculosis, which produces 1-D-*myo*-inosityl-2-deoxy-2-(N-acetyl-L-cysteinyl)amino- α -D-glucopyranoside (or mycothiol) as principal thiol antioxidant (Spies HSC and Steenkamp DJ (1994) Thiols of

intracellular pathogens: Identification of ovothiol A in *Leishmania donovani* and structural analysis of a novel thiol from *Mycobacterium bovis* (BCG). *Eur.J.Biochem.* 224, 203 – 213; Fahey RC (2001) Novel thiols of prokaryotes. *Annu Rev Microbiol.* 55, 33 – 356), and in trypanosomatids which cause a wide spectrum of maladies, ranging from South American Chagas' disease to the various forms of New World and Old World leishmaniases. Trypanothione reductase, a member of the disulphide reductase family of enzymes, proved to be essential for the survival of trypanosomatids within the host (Grieger S, Schwarz W, Ariyanayagam MR, Fairlamb AH, Krauth-Siegel RL and Clayton, C. (2000) Trypanosomes lacking trypanothione reductase are avirulent and show increased sensitivity to oxidative stress. *Mol Microbiol* 35, 542 – 52; Tovar J, Wilkinson S, Mottram JC, Fairlamb AH (1998) Evidence that trypano-thione reductase is an essential enzyme in *Leishmania* by targeted replacement of the tryA gene locus. *Mol.Microbiol* 29, 653 – 60; Dumas C, Quellette M, Tovar J, Fairlamb AH, Tamar S, Olivier M and Papadopolou B. Disruption of the trypanothione reductase gene of *Leishmania* decreases its ability to survive oxidative stress in macrophages. *EMBO J.* 16, 2590 – 8). Similar experiments have not yet been performed in the mycobacteria.

Procedures for the diagnosis of tuberculosis, based on the immunochemical detection of mycothiol, are known but research in this area is hampered by the lack of availability of mycothiol. Mycothiol and ovothiol A, a mercaptohistidine which has been identified in trypanosomatids, are, for example, not available from commercial sources.

Chemical synthesis of the principal low molecular mass thiol from the Mycobacteria, viz. mycothiol, has proven to be difficult and costly, and only small amounts of the compound could be isolated from natural

sources, ie. the mycobacteria (Spies HSC and Steenkamp DJ (1994) Thiols of intracellular pathogens: Identification of ovothiol A in *Leishmania donovani* and structural analysis of a novel thiol from *Mycobacterium bovis* (BCG). *Eur.J.Biochem.* 224, 203 - 213). The purification of thiol compounds from natural sources is complicated by their reactivity. Following their extraction thiols are readily oxidized to symmetrical and mixed-disulphides. Thiols are especially unstable in mixtures where they are not only subject to atmospheric oxidation but also undergo addition reactions to electrophiles, such as aldehydes and activated double bonds. It is thus important to separate the thiol species from contaminants as soon as possible after extraction, or to convert them, in a manner that can readily be reversed, to a more stable form, such as the disulphide. It is an object of the invention to address this problem.

Accordingly the invention provides a method of isolating a thiol R'SH from a thiol-containing mixture, the method including the steps of forming a mixed disulphide R'SSR of the thiol R'SH in the mixture, in which R is a non-immobilised hydrophobic moiety; purifying the mixed disulphide R'SSR; reducing the purified mixed disulphide R'SSR to produce a mixture of the thiols R'SH and RSH; and isolating the thiol R'SH.

Purifying the mixed disulphide R'SSR may include exploiting its increased hydrophobicity relative to the thiol R'SH, using methods such as, for example, reversed phase chromatography and selective precipitation.

Forming the mixed disulphide may include reacting the free thiol species with a mixed disulphide compound $R'SSR$ in which R' is a 2-thiopyridyl group and R is the non-polar thiol group.

5 The mixed disulphide may be purified by means of reversed phase high performance liquid chromatography (HPLC).

The purified disulphide may be reduced with dithiothreitol or β -mercaptoethanol.

The thiol $R'SH$ may be isolated by high performance liquid chromatography (HPLC).

10 The HPLC may be performed on a C18 reversed phase medium having a polar mobile phase, such as, for example, water and/or acetonitrile.

The group R may be a substituted or unsubstituted polynuclear aromatic group. In particular, it may be a 6-hydroxynaphthyl group. The
15 mixed disulphide may thus be 2-thiopyridyl-6-hydroxynaphthyl disulphide.

The thiol $R'SH$ may be 1-D-*myo*-inosityl-2-deoxy-2-(N-acetyl-L-cysteiny)amino- α -D-glucopyranoside, or mycothiol. This thiol is produced by the bacterium *Mycobacterium tuberculosis*. The disulphide may accordingly
20 be 2-S-(mycothioly)l-6-hydroxynaphthalenedisulphide.

According to another aspect of the invention, there is provided a disulphide of the formula $R'SSR$ in which $R'S$ is mycothioly and R of the substituent RS is a hydrophobic moiety.

R may be a polynuclear aromatic group. It may, in particular, be the 6-hydroxynaphthyl group.

The invention will now be described, by way of example, with reference to the accompanying non-limiting examples and diagrammatic drawings.

In the drawings,

Figure 1 shows the results of a spectrophotometric titration of 2-S-(2'-thiopyridyl)-6-hydroxynaphthyl disulphide in 25mM triethylammonium-carbonate, pH 8.1, with dithiothreitol;

Figure 2 shows absorbance spectra of (A) the 2-thio-6-hydroxynaphthalene thiolate anion (solid line) and of 6-hydroxy-2-naphthyl disulphide (broken line), (B) 2-thio-6-hydroxynaphthalene at pH 8.2 in 25mM potassium phosphate buffer (solid line) and at pH 3.9 in 25mM triethylammonium acetate (broken line);

Figure 3 shows the dependence of the recovery of glutathione as 2-S-(glutathionyl)-6-hydroxynaphthalene on the ratio of glutathione to 2-S-(2'-thiopyridyl)-6-hydroxynaphthalene. 100µl of 0.66mM glutathione in a mixture containing 4 parts acetonitrile and 6 parts of 0.1M sodium acetate, pH 4.8, was mixed with different amounts of 2-S-(2'-thiopyridyl)-6-hydroxynaphthyl disulphide and incubated overnight at room temperature. The mixtures were then all adjusted to a volume of 0.5ml. Aliquots were diluted with one part of water and injected onto a Phenomenex C18 reversed phase column (250 X 4.6mm);

Figure 4 shows an analytical HPLC of a perchloric acid extract of *Mycobacterium smegmatis* after treatment with 2-S-(2'-thiopyridyl)-6-hydroxynaphthyl disulphide for 100min. Mycothiol; 2-S-(mycothiolyl)-6-

hydroxynaphthylidysulphide, reagent; 2-S-(2'-thiopyridyl)-6-hydroxynaphthylidysulphide, naphthylidS; 6-hydroxy-2-naphthyl-disulphide;

Figure 5 shows the structure of 2-S-(2'-thiopyridyl)-6-hydroxynaphthylidysulphide; and

5 Figure 6 shows the structure of Mycothiol.

EXAMPLE 1

Synthesis of 2-S-(2'-thiopyridyl)-6-hydroxynaphthylidysulphide:

100 mg 6-hydroxy-2-naphthylidysulphide (obtained from Sigma-Aldrich S.A.) was dissolved in 50ml of a 1:1 mixture of acetonitrile and water and was reduced by the addition of 300mg sodium borohydride at room temperature. Reduction resulted in the appearance of a yellow colour due to the formation of the thiolate anion of 2-thio-6-hydroxynaphthalene. (The molar extinction coefficient, ϵ_{298} , was estimated to be $18.5 \text{ mM}^{-1}\text{cm}^{-1}$ by recording the absorbance spectrum of an aliquot in 25mM K_2HPO_4). The borohydride was destroyed by the addition of 1ml glacial acetic acid and the pH of the resulting solution adjusted to 4.6 by the addition of dipotassium phosphate. (The pH of 4.6 was chosen to be significantly below the pK_a of 2-thio-6-hydroxynaphthalene, which was estimated to be approximately 6.4 by spectrophotometric titration.) The 2-thio-6-hydroxynaphthalene solution was then added dropwise and with stirring over a period of 20 minutes to 25 ml of a solution containing 237mg 2,2'-dithiodipyridyl (obtained from Sigma-Aldrich S.A.) in the same solvent. The mixture was left for 20 minutes and was then diluted with one part of water and cooled to 4°C . The resultant precipitate was collected by centrifugation and was dissolved in a 50% acetonitrile/ water mixture.

The precipitate consisted principally of 6-hydroxy-2-naphthyldisulphide and 2-S-(2'-thiopyridyl)-6-hydroxynaphthyldisulphide (for structure thereof, see Figure 5). These components were separated on a Preparative Vydac TP1022 reversed phase HPLC column using a linear gradient over 40 minutes from 50% B to 100% B (A: 0.1% trifluoroacetic acid; B: 100% acetonitrile). It will be appreciated that the starting material, 6-hydroxy-2-naphthyldisulphide, obtained from this step can be recycled.

EXAMPLE 2

Use of 2-S-(2'-thiopyridyl)-6-hydroxynaphthyldisulphide:

Mycobacterium smegmatis was cultured and was harvested in the late log phase of growth. 4g (wet weight) of packed cells were suspended in 8ml of a solution containing 2mM EDTA and 0.75 M perchloric acid in a 1:1 mixture of acetonitrile and water. The cells were disrupted by sonication and the mixture clarified by centrifugation. The pH of the clear supernatant was adjusted to 4.8 by the addition of solid potassium carbonate and the potassium perchlorate precipitate was removed by centrifugation. The resulting mycobacterial extract contained 8 μ mole of dithiopyridyl reactive material in a volume of 9.3 ml. Mycothiol was purified from this extract by the following steps.

Step 1: Acetonitrile (6.2ml) was added to the mycobacterial extract. A slight precipitate developed which was again clarified by centrifugation. The mycobacterial extract was subsequently added dropwise with stirring to 3.65 ml of a 60% v/v acetonitrile solution containing 16 μ moles of 2-S-(2'-thiopyridyl)-6-hydroxynaphthyldisulphide. Formation of S-2-

(mycothioly)-6-hydroxynaphthalenedisulphide was monitored by HPLC analysis of aliquots of the reaction mixture (see below).

Step 2: After 3.5h the mixture was diluted 5-fold with water and was then passed through a SepPak C18 cartridge. The cartridge was washed with water. S-2-(mycothioly)-6-hydroxynaphthyldisulphide was retained and could be eluted using a minimal quantity of 50% v/v acetonitrile/water. S-2-(mycothioly)-6-hydroxynaphthyldisulphide can be detected in the effluent fractions by TLC (see below). The SepPak step results in a large purification and concentration of the derivatized mycothiol is achieved.

Step 3: The acetonitrile content of the solution was subsequently reduced to less than 10% under a stream of nitrogen and the solution was chromatographed on a Preparative Vydac TP1022 C18 reversed phase column using the following conditions: 10mins at 100%A, followed by a gradient from 0 – 100%B over 40min. at a flowrate of 4ml/min (A: 0.1%trifluoroacetic acid; B: acetonitrile). S-2-(mycothioly)-6-hydroxynaphthalenedisulphide was eluted at 35.5 min as the principal component and only lesser contaminants are removed in this step. The concentration of 2-S-(mycothioly)-6-hydroxynaphthyldisulphide can be estimated by recording the absorbance spectrum of an aliquot in 25mM K_2HPO_4 after reduction with 1mM dithiothreitol and using $\epsilon_{298} = 18.5mM^{-1}cm^{-1}$. 1.43 μ moles of mycothiol was recovered as 2-S-(mycothioly)-6-hydroxynaphthyldisulphide.

Step 4: Reduced mycothiol (for structure thereof, see Figure 6) was obtained by reduction of S-2-(mycothioly)-6-hydroxynaphthyldisulphide with dithiothreitol and separation on the same preparative column, using the

following conditions: 10 min at 100%A followed by a 40 min gradient to 25%B (A: 0.1%trifluoroacetic acid; B: 100% acetonitrile) at a flowrate of 4.0ml/min.

It will be appreciated that, when conducted on a larger scale, steps in which acetonitrile is removed under a stream of nitrogen can be replaced
5 by vacuum distillation using a rotary evaporator.

Because of the large difference in polarity between mycothiol and mixed disulphides containing 2-thio-6-hydroxynaphthalene, considerable simplification of the above steps is possible. Instead of diluting the reaction mixture in Step 2, acetonitrile can be evaporated under a stream of nitrogen
10 or by means of a rotary evaporator. This results in precipitation of 2-S-(2'-thiopyridyl)-6-hydroxynaphthalene disulphide and any 6-hydroxy-2-naphthylidisulphide which might have formed, while 2-S-(mycothioly)-6-hydroxynaphthylidisulphide remains in solution. Moreover, mycothiol can be obtained directly from the latter after Step 2, by reduction with 2-
15 mercaptoethanol and extraction of the excess reductant with ethylacetate. Upon repeating the SepPak separation mycothiol will not be adsorbed, while 2-thio-6-hydroxynaphthalene and more apolar contaminants which eluted from the SepPak cartridge in 50% acetonitrile will be selectively retained.

Analysis of reaction progress:

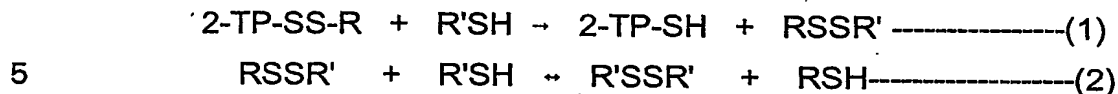
20 The formation of the reagent 2-S-(2'-thiopyridyl)-6-hydroxynaphthylidisulphide and of 2-S-(mycothioly)-6-hydroxynaphthalenedisulphide was followed by HPLC on a 4.6 X 250mm Phenomenex C18 reversed phase column using the same gradient conditions as for the chromatography of 2-S-(mycothioly)-6-
25 hydroxynaphthalenedisulphide described above (ie. on the Vydac TP1022

column), but with a lower flow rate of 0.8ml/min. The eluate was monitored at 280nm and the concentrations of the components estimated from the integrated peak areas. In the case of 2-S-(mycothioly)-6-hydroxynaphthalenedisulphide the concentration of the compound in a solution, used to establish the necessary calibration curve, was determined with reference to the molar extinction coefficient of 2-thio-6-hydroxynaphthalene, which is liberated upon reduction, while pyridine-2-thione and 2-thio-6-hydroxynaphthalene served as standards for determination of the concentration of 2-S-(2'-thiopyridyl)-6-hydroxynaphthyl-disulphide.

2-S-(2'-thiopyridyl)-6-hydroxynaphthyl-disulphide and 6-hydroxynaphthyl-disulphide are well-separated by TLC on silica gel G60 containing fluorescent indicator. The developing solvent was acetone: 0.1M Na-acetate:: 4:6 (Rf values of 0.5 and 0.24, respectively). 2-S-(mycothioly)-6-hydroxynaphthalenedisulphide can be resolved from 2-S-(2'-thiopyridyl)-6-hydroxynaphthyl-disulphide and 6-hydroxynaphthyl-disulphide on cellulose containing fluorescent indicator using butanol:acetic acid:water:: 4:2:2: as developing solvent (Rf = 0.7 for 2-S-(mycothioly)-6-hydroxynaphthalenedisulphide while 2-S-(2'-thiopyridyl)-6-hydroxynaphthyl-disulphide and 6-hydroxynaphthyl-disulphide moved with the solvent front).

The fact that thiols form mixed disulphides with the thiopropyl group rather than with 2-thiopyridine, can be attributed to the favourable energy of ionization of 2-thiopyridine and the delocalization of charge on the thiolate anion into the pyridine ring. In the present invention, the thiol species to be isolated is reacted with a mixed disulphide between 2-thiopyridine and a non-polar thiol with significantly higher pK_a of the thiol group than is the

case for 2-thiopyridine. At a pH intermediate the pK_a of the non-polar thiol and that of 2-thiopyridine the reaction of a thiol with the 2-thiopyridyl mixed disulphide is strongly biphasic:



In these reactions 2-TP represents 2-thiopyridyl, R is a non-polar thiol group and R'S a naturally occurring thiol species. The equilibrium in reaction (2) should preferably be far to the left.

In the above example it has been shown that 2-thio-6-hydroxynaphthalene satisfies the requirements for the non-polar thiol and generates a mixed disulphide, RSSR', which can be conveniently adsorbed and concentrated onto reversed phase materials. This greatly facilitates their isolation and purification and the compounds of interest can be recovered by reduction with an excess of dithiothreitol or β -mercaptoethanol. Moreover, the thiolate anion of 2-thio-6-hydroxynaphthalene has characteristic absorbance bands which are convenient for the quantification of the amount of thiol with which it has associated to form a mixed disulphide.

The reduction of 2-S-(2'-thiopyridyl)-6-hydroxynaphthyl disulphide by thiols, including dithiols such as dithiothreitol, proceeds in the biphasic manner set out above, with the initial release of pyridine-2-thione and the intermediate formation of a mixed disulphide of the reducing thiol with 2-thio-6-hydroxynaphthalene (Fig.1). In Fig.1 the initial increase in absorbance at 343nm can be attributed principally to the formation of pyridine-2-thione, while the subsequent decrease is due to the formation of the 2-thio-6-hydroxynaphthalene thiolate anion which has a

lower absorbance as compared to the homo- and heterodisulphides at this wavelength (see Fig.2A). With glutathione as the reducing thiol the formation of 2-S-(glutathionyl)-6-hydroxynaphthalenedisulphide is practically stoichiometric at pH values less than 5. The chromatographic behaviour of such mixed disulphides is governed to a large extent by the presence of the naphthol moiety. They are tightly bound on C18 reversed phase cartridges, but are eluted by water-miscible organic solvents. The amount of pyridine-2-thione released in the initial reaction can be quantified due to the absorbance of pyridine-2-thione at 343nm ($\epsilon_{298} = 8.08 \text{ mM}^{-1}\text{cm}^{-1}$) and the amount of mixed dithiol formed can be estimated from the intense absorbance of the thiolate anion ($\text{pK}_a \sim 6.5$) of 2-thio-6 hydroxynaphthalene ($\epsilon_{298} \sim 18.5 \text{ mM}^{-1}\text{cm}^{-1}$) which is formed when the mixed disulphide is reduced with dithiothreitol.

Spectra of oxidized and reduced 2-thio-6-hydroxynaphthalene are presented in Fig.2A and in its protonated and thiolate forms in Fig.2B:

In order to establish optimal conditions for the conversion of thiols to mixed disulphides by reaction with 2-S-(2'-thiopyridyl)-6-hydroxynaphthylidysulphide, glutathione was incubated overnight at room temperature with various amounts of the reagent. As shown in Fig.3, the yield of 2-S-(glutathionyl)-6-hydroxynaphthalenedisulphide reaches a plateau at a ratio of about 3 moles of reagent per mole of glutathione. At a ratio of reagent to glutathione of 1.7, the conversion of glutathione to 2-S-(glutathionyl)-6-hydroxynaphthalenedisulphide reached a level of 72.7% after 100min, showed a slight increase to 75.4% at 175 min and declined to 67.86% when the mixture was left overnight, presumably also because of thiol-disulphide equilibria which are established slowly between pyridine-2-thione and the mixed disulphides. This explanation was also supported by a significant increase in the integrated peak area for 6-hydroxy-2-

naphthyldisulphide which is evidently a side product. At higher ratios of reagent to glutathione it is evident that a recovery of at least 75% of glutathione as 2-S-(glutathionyl)-6-hydroxynaphthalenedisulphide should be attainable.

5 The HPLC profile of a mixture which contained a perchlorate extract of *M. smegmatis* and 2-S-(2'-thiopyridyl)-6-hydroxynaphthyldisulphide 3.6-fold in excess of 4,4'-dithiodipyridyl reactive material is shown in Fig.4. The reagent clearly has a high degree of specificity for thiols as evidenced by the prominence of the peak due to 2-S-(mycothioly)-6-
10 hydroxynaphthyldisulphide at 21.9mins. The isolation of mixed disulphides containing the 2-thio-6-hydroxynaphthalene chromophore can be followed either by HPLC of aliquots or by TLC, in each case without any need for derivatization and the HPLC result can be used for quantification.

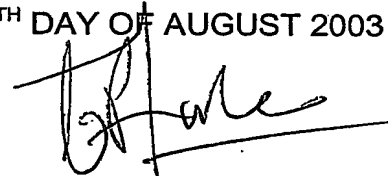
15 The method described can be scaled up to allow for the purification of significant amounts of mycothiol. Approximately 600g (wet weight) of *M. smegmatis* would be required for the isolation of 100mg of mycothiol and extraction of this amount of cells with perchloric acid will generate 1.5 - 2 litres of extract. Converting the mycothiol in the extract to 2-S-(mycothioly)-6-
20 hydroxynaphthyldisulphide, which can be selectively adsorbed and concentrated onto reversed phase material, would require only a three-fold excess of reagent over mycothiol and the success of the reaction can be directly evaluated by analytical HPLC as shown in Fig.4.

25 The intermediates in the mycothiol biosynthetic pathway, such as, for example, 1-D-*myo*-inosityl-2-deoxy-2-amino- α -D-glucopyranoside (α -D-GI), are also not commercially available, but are accessible from mycothiol. Mycobacteria possess a deamidase which specifically cleaves alkylated

mycothiol to α -D-Gl and a mercapturic acid, which is excreted from the cells (6). The Inventor believes that α -D-Gl could be a convenient starting point for the synthesis of inhibitors of the mycothiol biosynthetic pathway.

5 The invention provides an improved and more cost-effective method for the isolation and purification of low molecular mass thiols from natural sources. It is an advantage of the invention illustrated, that the mixed disulphide has a hydrophobic character which facilitates their isolation and purification. The compounds of interest can then be recovered by reduction with an excess of dithiothreitol or β -mercaptoethanol. It is a further advantage
10 that the method in accordance with the invention converts thiols to stable disulphides at an early stage in the isolation process and the greater part of purification is achieved in the first step of the method, which step can readily be scaled up. Furthermore, it dispenses with the need for the use of activated thiopropyl resins which are expensive to buy and which necessitate further
15 purification steps to recover the thiols from the eluate of such columns.

DATED THIS 27TH DAY OF AUGUST 2003



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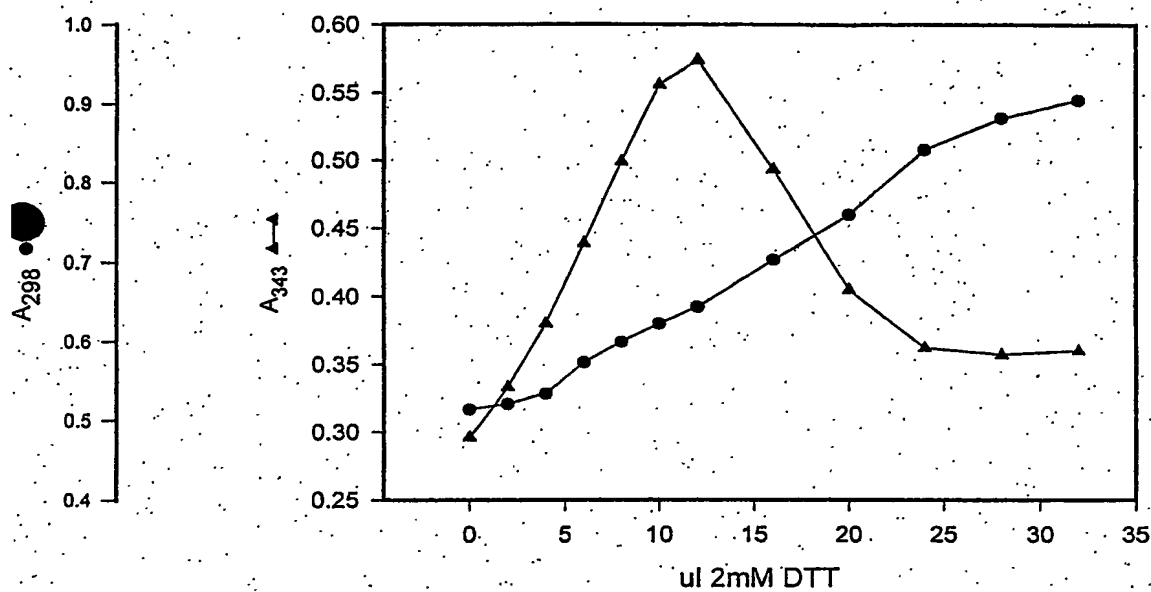


FIGURE 1

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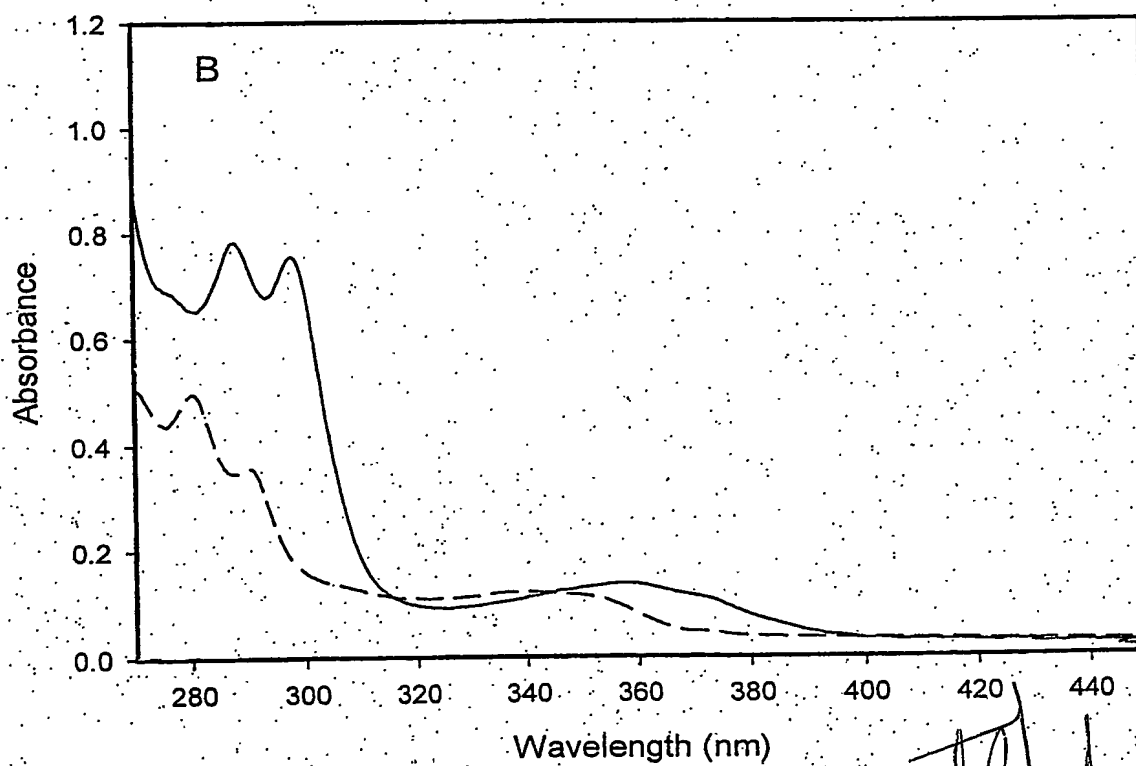
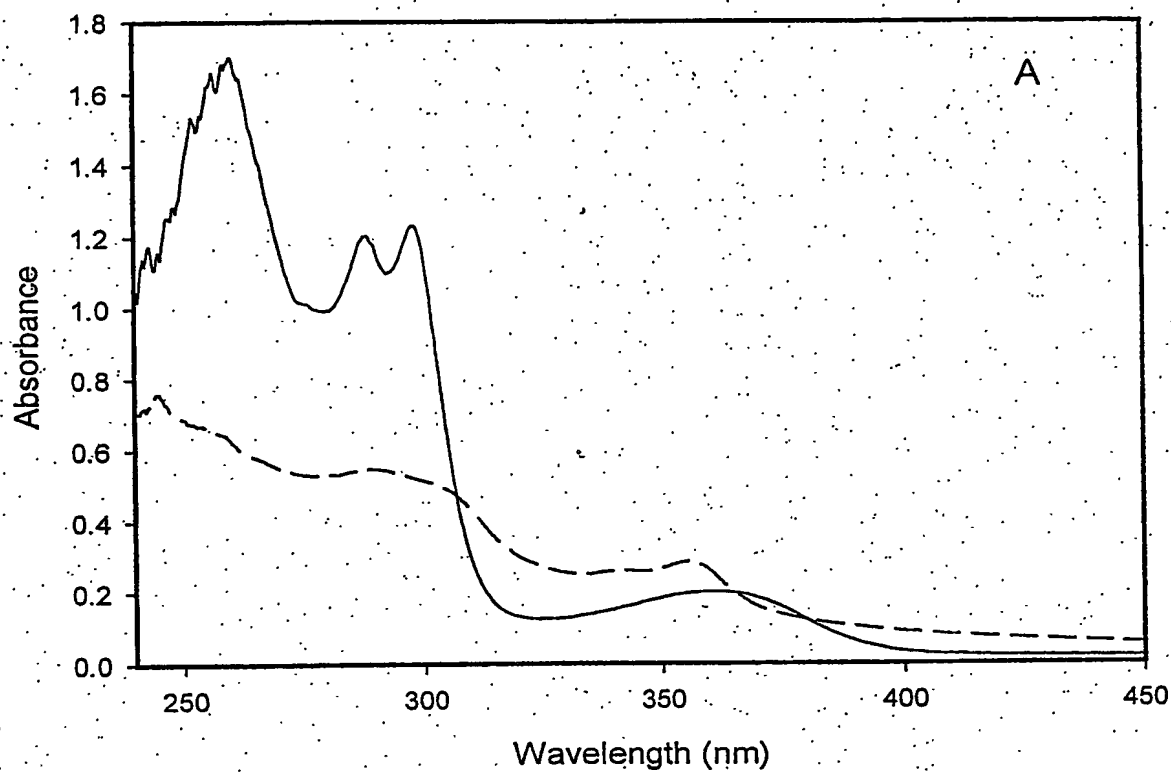


FIGURE 2

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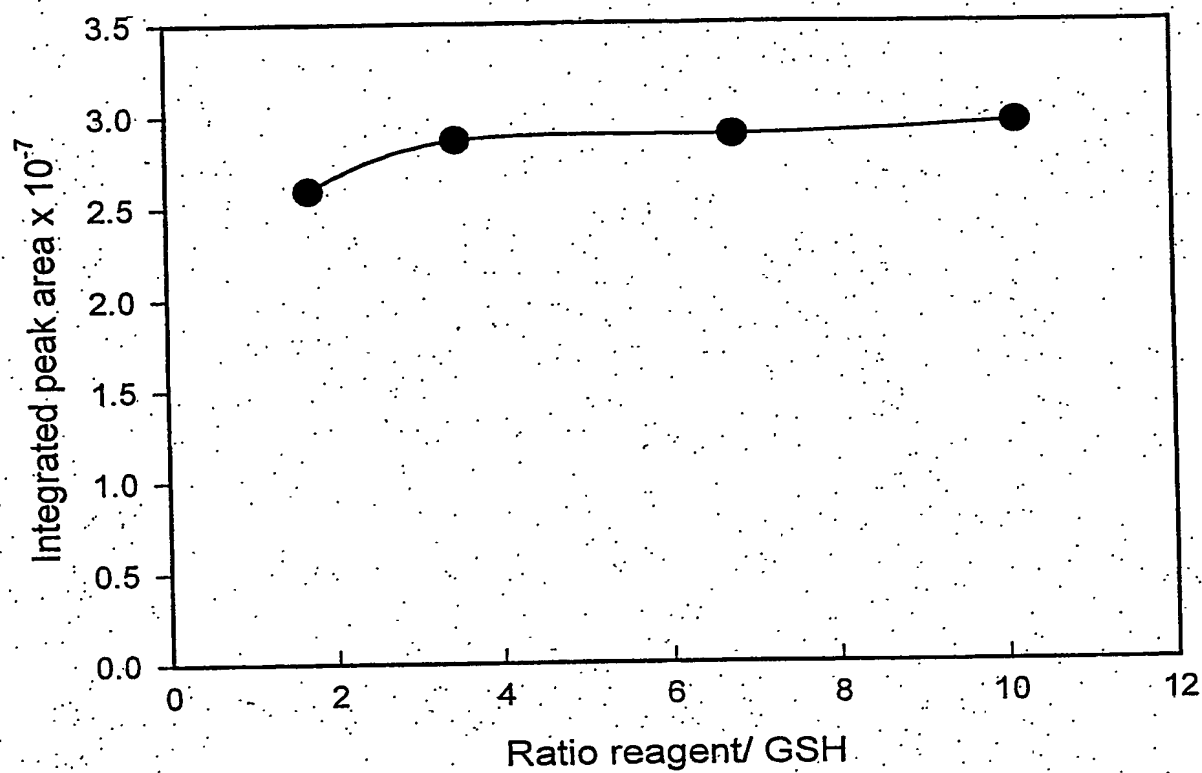


FIGURE 3

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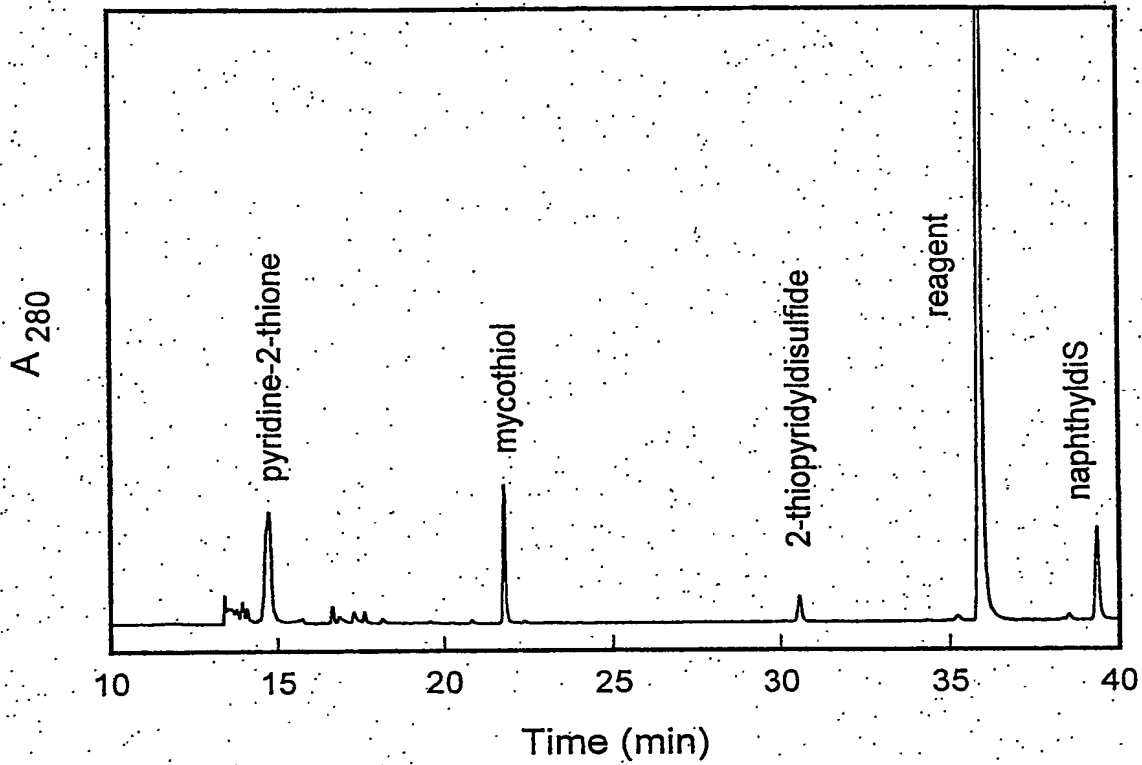


FIGURE 4

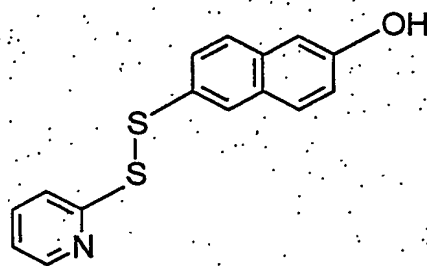


FIGURE 5

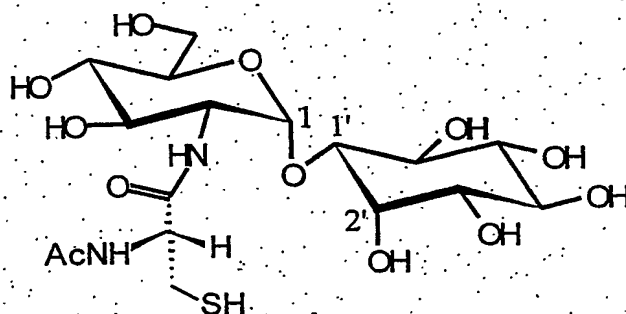


FIGURE 6

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